{Exhibit 34}

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S lid-Phase Enzyme Immunoassay for Herpes Simplex Virus

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An enzyme immunoassay was used f r the rapid detection of herpes simplex virus type 1 (HSV-1). The test utilized specific antibodies to HSV-1 attached to a solid phase. A laboratory preparation of HSV-1 was detectable in amounts of ≥50 50% tissue culture-infective doses by this method. Tests performed with clinical samples indicated a specificity of 95%. The sensitivity appeared to depend on the length of time the samples were stored. When samples were stored frozen for three to six months, the sensitivity was 46% of that of tissue culture isolations performed when the samples were first collected. The sensitivity was 89% in comparison with attempts at tissue culture reisolation with the same samples at the time of enzyme immunoassay. The immunoassay also detected HSV in seven of 10 positive samples that were stored frozen for no more than two weeks before testing.

The herpes simplex viruses (HSV) are becoming increasingly important etiologic agents in clinical virology. On the basis of biological characteristics, these viruses have been divided into two types, HSV-1 and HSV-2 [1, 2]. Both types infect various human organs, causing a variety of diseases. HSV has been isolated from ocular infections [3], fever blisters, gingivostomatitis, skin eruptions, male and female genitals [4, 5], and brain [6], from immunosuppressed patients [7], and from newborn infants [8]. Recent evidence [9] has supported the link between HSV-2 and cervical cancer. In view of the important clinical implications of these viruses, a rapid, easily performed, sensitive, and specific test for the detection of the virus is vital to effective diagnosis and patient management. Classical procedures do not completely meet these criteria.

The most commonly used method for the detection of HSV is isolation in tissue cultures. Many varieties of tissue culture cells support the growth of these viruses. Human embryonic lung and kidney, primary human amnion, and BHK-21 (baby hamster kidney) cells have been reported to be highly susceptible [10–12]. HeLa, RK-13 (rabbit kidney), and Vero cells are also used in some laboratories. Although tissue culture is the conventional method of detection and identification of virus, this method is expensive and time-consuming.

During the last 10–15 years, attempts were made to simplify the tissue culture procedure. Microtechniques with use of cell cultures in microtiter plates were developed for a variety of viruses [13–15]. The quantal microneutralization test described by Pauls and Dowdle [14] in 1967 is probably the most reliable and reproducible of the methods, but it is relatively cumbersome and time-consuming. The simplified microtiter test for HSV described by Stalder et al. [15] expresses the results in a simpler fashion, namely, as end point neutralization titers corrected to a standard input of virus.

Several immunofluorescence tests, both direct and indirect, have been described for the detection of HSV antigens and antibodies [16–18]. The techniques are rapid, sensitive, and specific, but they require highly trained technicians and the use of tissue cultures. Radioimmunoassays for typing HSV isolates and HSV antibodies in human sera have been reported by Forghani et al. [19, 20]. Results obtained by radioimmunoassay typing of HSV antibodies showed good agreement with those obtained by microneutralization. An indirect immunoperoxidase method for typing of HSV strains has also been reported by Benjamin [21]. There was complete agreement between the results obtained by immunoperoxidase and kinetic neutralization.

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The present report describes the development of and our own experience with a rapid enzyme immunoassay (enzyme-linked immunosorbent assay, ELISA) for detecting HSV.

Materials and Methods

Viral strains. The MacIntyre strain was used as a reference strain of HSV-1, and the MS strain was used as a reference strain of HSV-2. The strains were purchased from the American Type Culture Collection (Rockville, Md.).

Antibodies. Specific antibodies to HSV-1 and HSV-2 prepared in rabbits were obtained from Dr. A. Martin Lerner, Wayne State University School of Medicine, Detroit, Mich. Additional antibodies to HSV-1 prepared in rabbits were obtained from Dr. A. Kaplan, Vanderbilt University School of Medicine, Nashville, Tenn.

Tissue cultures. Vero and RK-13 cells (Flow Laboratories, Rockville, Md.) were used for the propagation and titration of HSV-1 and HSV-2 and for the isolation of virus from clinical samples. Microtiter plates (Cooke Laboratory Products, Alexandria, Va.) were used for titrations of virus and for clinical isolations.

Clinical samples. One hundred two clinically defined samples, collected from various sites of infection, were obtained from Harbor General Hospital, Torrance, Calif. Ten of the 102 samples were frozen for only two weeks at −90 C, and the rest were frozen for from three to six months at −90 C. The samples were processed in tissue culture at the time of collection and were identified as positive or negative on the basis of viral isolations.

Normal samples for simulated clinical trial. Fifty throat swabs were collected from healthy individuals working at the Organon facility at El Monte. The swabs were each placed in 2 ml f Hanks' solution and were frozen overnight at −70 C. On the next day the samples were thawed, and 0.9 ml from each sample was transferred to a sterile tube. The samples were then divided into two batches. The 50 tubes in one batch each contained about 1.1 ml of Hanks' soluti n and were used as a control batch. The other batch consisted of 50 tubes, each containing 0.9 ml of the Hanks' solution. This test batch was later randomly divided into 10 groups of five tubes each. To each group of five tubes was added HSV-1 diluted twofold from 1:2 t 1:512 (12.5 $TCID_{50}$ to 6,400 $TCID_{50}$ per ELISA procedure). The 10 groups of simulated samples were then coded and tested by ELISA. The batch of normal samples was tested by ELISA on the next day. Both the simulated and normal samples were tested in tissue cultures.

ELISA. Polystyrene beads, 2 mm in diameter, were sensitized by adsorption with rabbit γ-globulins isolated by precipitation with $(NH_4)_2SO_4$. After drying, the sensitized beads were assembled into the reaction containers used in the test. These test containers, IncuFilters® (Organon Diagnostics), were molded in two pieces out of styrene acrylonitrile (figure 1). After 25 sensitized beads were added to the bases, the bases were joined by ultrasonic welding to the tubes containing the "fingers" that hold the beads in place. After assembly of tubes (figure 2), plastic caps were fitted to the IncuFilters. Each cap contained a small hole through which all wash fluids passed.

Enzyme-antibody conjugates were prepared by coupling of horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.) to the rabbit anti-HSV-1 γ-globulin preparations with glutaralde-

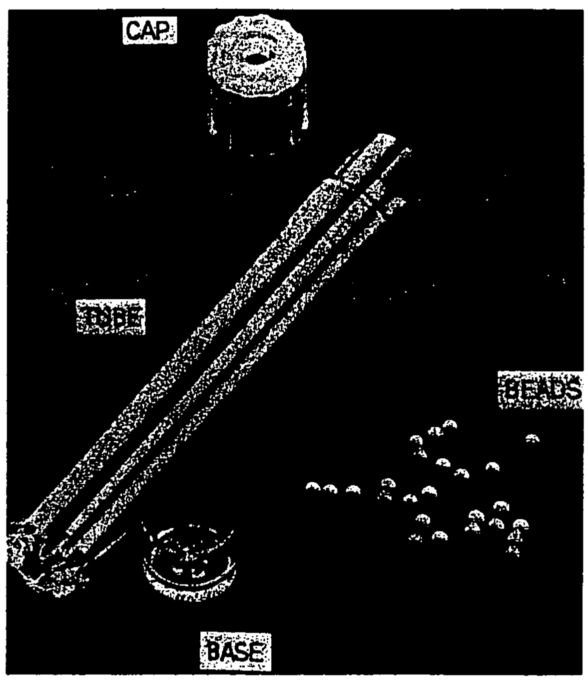

Figure 1. IncuFilter® before assembly.

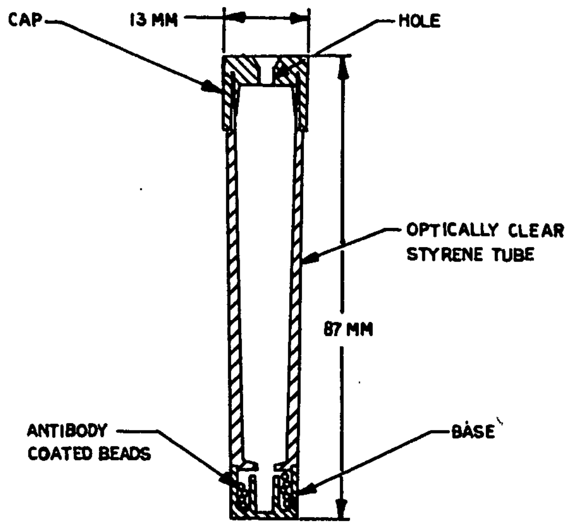

Figure 2. Diagram of assembled IncuFilter.®

hyde. The procedure used was similar to that described by Avrameas [22]. The optimal dilution of conjugate to be used in the test was determined by titration.

The ELISA was performed by incubation of 0.2 ml of the sample in the IncuFilter for 2 hr in a 37 C water bath. A wash fluid (2 ml) consisting of 0.2 M Tris, 0.2 M NaCl, and 0.05% Triton N–101 (Rohm and Haas, Philadelphia, Pa.), pH 7.4, was added to each IncuFilter. The wash fluid and the sample fluid were then aspirated with a cannula connected to a vacuum source and a trap containing disinfectant. Conjugate (0.2 ml) was then added through the cap orifice by means of a modified 10-ml repetitive syringe and a cannula. The IncuFilters were returned to the 37 C bath and incubated for 1 hr. The conjugate was removed by aspiration, and the IncuFilters were washed three times by adding 2.0 ml of wash fluid, mixing for 3–5 sec on a vortex-type mixer, and aspirating the fluid. After washing, a solution containing urea peroxide (substrate) and *o*-phenylenediamine (redox indicator) was added. Color was allowed to develop at room temperature in the dark for 1 hr, after which the reaction was stopped by the addition of 1 M citric acid. The absorbances of the solutions at 492 nm were determined directly in the IncuFilter with a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, N.Y.). Signal-to-noise (S:N) ratios were determined by comparing the absorbance of the test sample to that of a buffer control consisting of 0.05 M phosphate-buffered saline, pH 7.2 (PBS).

Results

ELISA assessment of negative population. For assessment of the feasibility of the solid-state ELISA for HSV, a negative sample was first surveyed to determine the negative cutoff level. This survey consisted of HSV samples in Hanks' balanced salt solution, taken by swabbing the inside of the cheeks and lower lip of laboratory personnel. Fifty samples were collected and tested by ELISA. In addition, the sample fluids were inoculated into microplate cultures of RK-13 cells. All were negative for viral CPE. A positive cutoff was taken as the mean (1.19) plus 2 SD, which was a S:N ratio of 1.57.

Assessment of dose response with a model system. Tests were performed in duplicate on serial twofold dilutions of a stock HSV-1 preparation diluted in PBS. At the same time, a $TCID_{50}$ titration was performed in microplate cultures of Vero cells. The infectivity of the stock preparation of virus was determined to be 3.2×10^5 $TCID_{50}$/ml. The results of ELISA (figure 3)

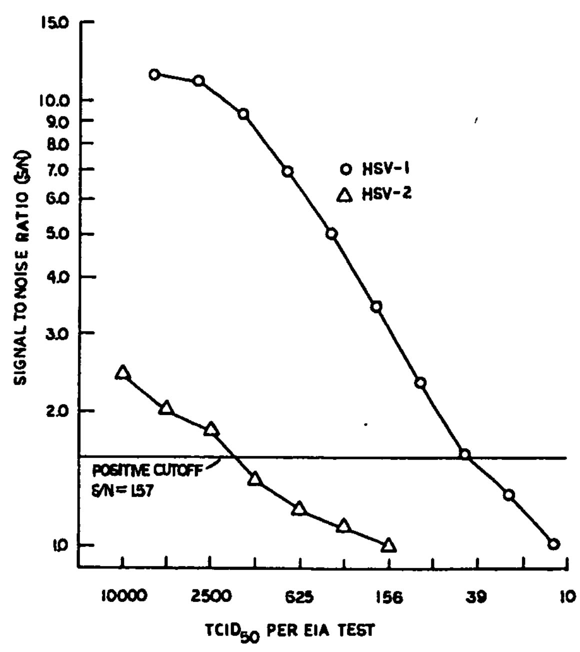

Figure 3. Reactions of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) in enzyme immunoassay (EIA) specific for HSV-1.

demonstrate the dose-response relation between the S:N ratio in ELISA and the $TCID_{50}$ concentration of HSV.

Specificity of ELISA for HSV-1. The specificity of ELISA for HSV-1 reagents was evident when dilutions of HSV-2 were tested. The HSV-2 preparation contained 10^5 $TCID_{50}$/ml, a level similar to that of the HSV-1 preparation mentioned above. However, when HSV-2 was tested, the ELISA end point was approximately 2,500 $TCID_{50}$ (figure 3).

Assay of simulated clinical samples. Simulated HSV-1-positive samples were prepared from aliquots taken from the negative samples (Organon). Twofold dilutions of the same stock HSV-1 preparation tested earlier were prepared in PBS. To 0.9 ml of randomly selected negative samples, 0.1 ml of a HSV dilution was added. Five different negative samples were used at each dilution of virus. The concentrations of virus at each dilution were identical to those tested earlier when PBS alone was used as the diluent. Each HSV-seeded positive sample was tested in duplicate by ELISA. The results (figure 4) indicated

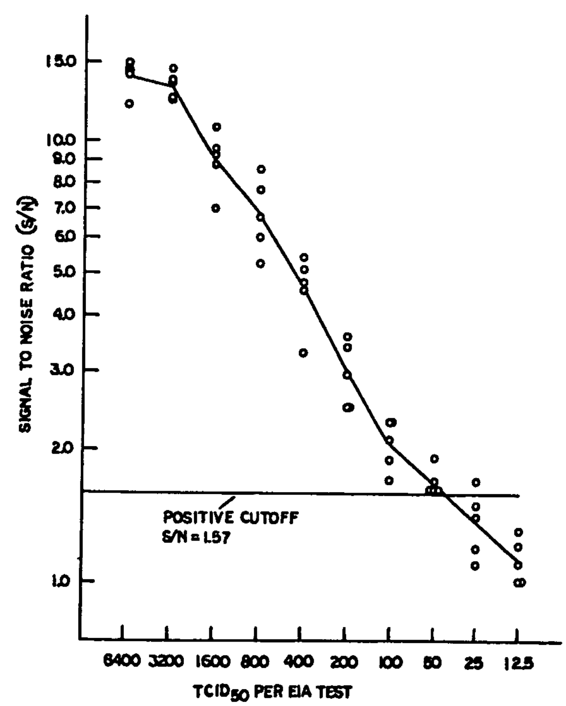

Figure 4. Detection by enzyme immunoassay (EIA) of herpes simplex virus that had been added to randomly selected mouth swab samples negative for virus.

Table 1. Average signal-to-noise (S:N) ratios in enzyme immunoassay of 53 clinical samples that were negative for herpes simplex virus in tissue culture isolations.

Sample site	No. of samples	S:N ratio
Wound	16	1.11
Throat	8	1.10
Female genital	5	1.06
Eye	6	1.23
Vesicle	5	1.12
Urine	3	1.17
Feces	2	1.05
Cerebrospinal fluid	2	1.25
Others	6	1.23

NOTE. Mean ± SD = 1.14 ± 0.15.

some variation at each concentration of virus. However, when the mean S:N ratio at each point was plotted, the results were similar to those of the test performed in PBS. With 1.57 as the positive cutoff S:N ratio, the ELISA detected all five samples containing 50 $TCID_{50}$ per test and one that contained 25 $TCID_{50}$.

Assay of clinical samples. Fifty-three of the 92 clinical samples were negative for HSV by tissue culture. A cutoff value above which ELISA would be considered positive was determined by ELISA of the tissue culture-negative samples (table 1); the cutoff was established as the mean plus 2 SD (S:N ratio, 1.44).

HSV was isolated initially from 39 of the 92 samples at Harbor General Hospital when the samples were fresh (figure 5). ELISA detected HSV in 18 (46%) of these samples after a lengthy storage. However, in attempts at reisolation by tissue culture, only nine samples were positive, and the ELISA detected HSV in eight (89%) of the nine samples. Eight of the samples that were negative by the reisolation in tissue culture but had been positive originally were still positive by ELISA. If these eight samples are not included as false-positives, the specificity remains as high as 95%. The data for these hospital samples are summarized in figure 5.

Effect of storage on ELISA response. Because frozen storage of the samples may have been detrimental to the ELISA response, 10 HSV-positive samples no more than two weeks old were obtained from Harbor General Hospital and tested by ELISA. These samples had also been stored frozen after the initial isolation and be-

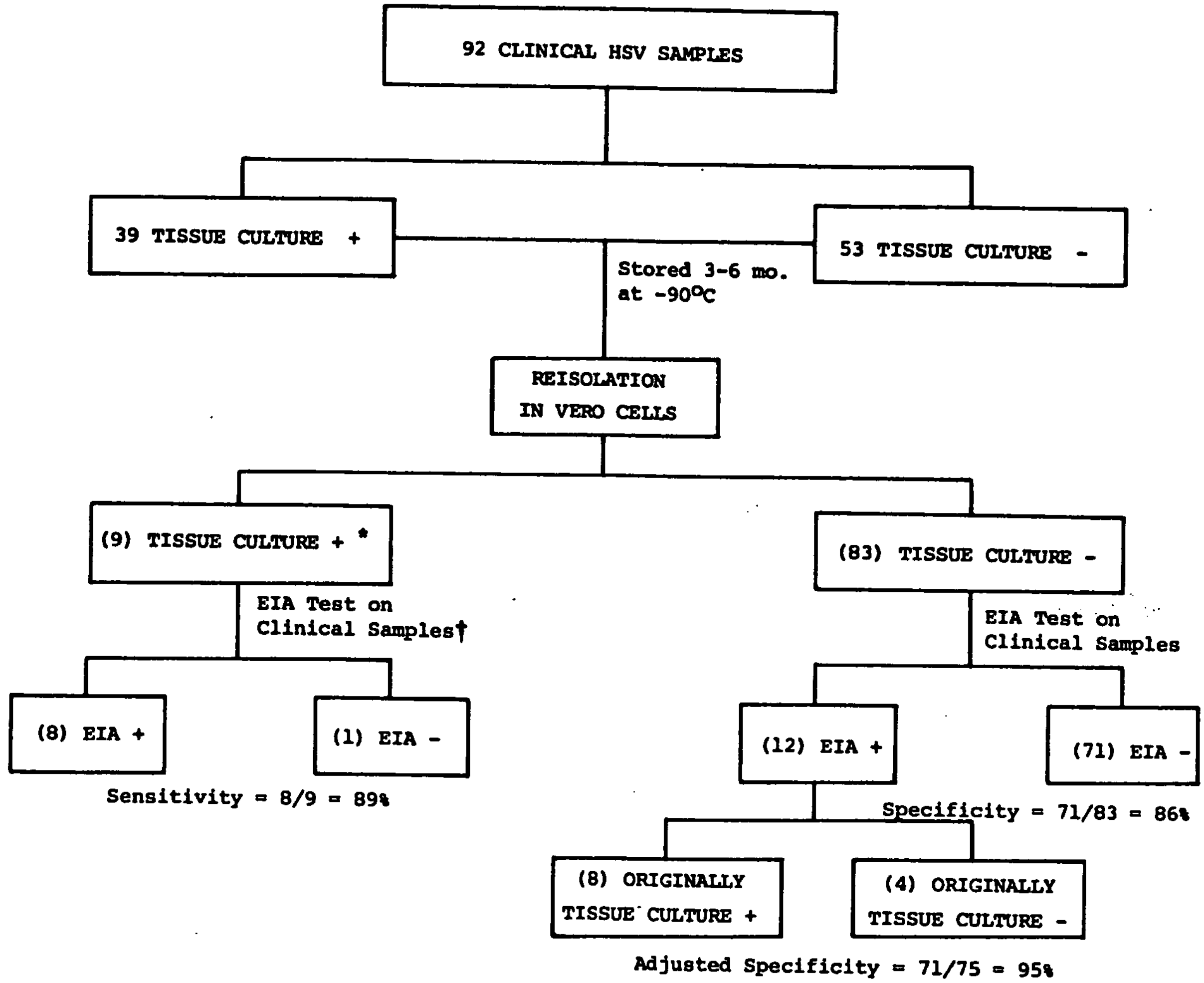

Figure 5. Results of enzyme immunoassay (EIA) for herpes simplex virus (HSV) in 92 clinical samples. * = measures HSV-1 and HSV-2; † = measures only HSV-1.

fore testing by ELISA. Tissue culture titrations on these samples showed no correlation between infectivity and response in ELISA (table 2). The ELISA was positive in seven of the 10 samples. The response with the fresh samples in this case appeared to be much better than the response with samples that had been stored for from three to six months.

Discussion

Although the ELISA was able to detect 50 $TCID_{50}$ of HSV-1 in the model systems described, it is by no means suggested that this level is an absolute value. Because the ELISA is an immunologic measurement of antigen present, the actual number of infective units may vary tremendously with many factors, for example, multiplicity of infection, temperature of incubation, and susceptibility of the cell line. The results do indicate, however, that an immunoassay for detection of HSV is feasible. It is anticipated that further refinements will result in a greater sensitivity than that reported here.

When ELISA results were compared to those from the original tissue culture isolations, it was evident that the age of a positive sample was very important in obtaining a positive response in ELISA. When samples had been stored for several months, the ELISA detected only 46% of those samples that were positive in the original isolation. However, when samples stored for a short period of time were tested, the ELISA was more sensitive. Admittedly, the number of sam-

Table 2. Results of enzyme-linked immunosorbent assay (ELISA) and $TCID_{50}$ titration of clinical samples positive for herpes simplex virus (HSV) that were stored frozen for one to two weeks.

Sample site	$TCID_{50}$/ELISA (HSV-1 or HSV-2)	S:N, HSV-1 ELISA*	ELISA result†
Cervix	1	1.6	+
Throat	4	3.2	+
Wound	10	9.5	+
Wound	10	6.3	+
Eye	10	1.8	+
Vesicle	32	1.2	–
Throat	64	1.7	+
Vesicle	64	1.3	–
Vagina	100	1.4	–
Vulva	200	1.5	+

*Signal-to-noise (S:N) ratio = absorbance of sample/absorbance of control.

†Positive S:N cutoff from negative clinical survey = 1.44.

ples in this later group was too small to permit any final conclusions.

The serologic types of the HSV-positive samples were not determined. Our data showed a reduced sensitivity of ELISA in detecting HSV-2. Our calculations would have been more meaningful if the positive samples had been typed.

The lack of correlation between infectivity and ELISA response in the clinical samples may be explained in two ways. First, the number of noninfective but antigenic particles probably varied greatly from sample to sample. Not all human cells respond in the same fashion; therefore, it is entirely possible that the ratio of infective particles to antigenic particles may vary widely from sample to sample. The lack of correlation between ELISA and titer of infectivity is further complicated by the possibility that this particular reagent system was specific for HSV-1. It is possible that some of the herpes simplex viruses isolated in these cases were HSV-2, in which case the apparent titer in ELISA would be low compared to the infectivity titer.

The solid-phase ELISA method appears to offer considerable promise as a rapid in vitro procedure for direct detection and typing of HSV antigen in clinical material. The sensitivity of the ELISA remains to be compared with the sensitivity of the immunofluorescence test. It appears that for fresh samples the sensitivity of the ELISA might approach that of viral isolation for diagnosis. The ease of performance and the minimal level of laboratory skills required make the ELISA a very desirable tool in the clinical diagnostic laboratory.

References

1. Plummer, G., Waner, J. L., Phuangsab, A., Goodheart, C. R. Type 1 and type 2 herpes simplex viruses: serological and biological differences. J. Virol. 5:51–59, 1970.
2. Marks-Hellman, S., Ho, M. Use of biological characteristics to type *Herpesvirus hominis* types 1 and 2 in diagnostic laboratories. J. Clin. Microbiol. 3:277–280, 1976.
3. Oh, J. O., Kimura, S. J., Ostler, H. B. Acute ocular infection by type 2 herpes simplex virus in adults. Arch. Ophthalmol. 93:1127–1129, 1975.
4. Josey, W. E., Nahmias, A. J., Naib, Z. M. Genital herpes simplex virus infection. Medical Aspects of Human Sexuality 7:114–139, 1973.
5. Dobin, R., Gill, F. A., Nahmias, A. J. Genital herpes simplex virus type 1 infection. Variability in modes of spread. J. Am. Ven. Dis. Assoc. 2:13–16, 1975.
6. Mayer, J. S., Bauer, R. B., Rivera-Olmos, V. M., Nolan, D. C., Lerner, A. M. *Herpesvirus hominis* encephalitis. Neurological manifestations and use of idoxuridine. Arch. Neurol. 23:438–450, 1970.
7. Whitley, R. J., Ch'ien, L. T., Dolin, R., Galasso, G. J., Alford, C. A. The collaborative study group. Adenine arabinoside therapy of herpes zoster in the immunosuppressed. NIAID Collaborative Antiviral Study. N. Engl. J. Med. 294:1195–1199, 1976.
8. Young, E. J., Killam, A. P., Greene, J. F. Disseminated herpesvirus infection. Association with primary genital herpes in pregnancy. J.A.M.A. 235:2731–2733, 1976.
9. Melnick, J. L., Courtney, R. J., Powell, K. L., Schaffer, P. A., Benyesh-Melnick, M., Dreesman, G. R., Anzai, T., Adam, E. Studies on herpes simplex virus and cancer. Cancer Res. 36:845–856, 1976.
10. Tokumaru, T. Herpesviruses. *In* E. H. Lennette and N. J. Schmidt [ed.]. Diagnostic procedures for viral and rickettsial infections. American Public Health Association, New York, 1969, p. 641–676.
11. Schmidt, N. J. Tissue culture in the laboratory diagnosis of viral infections. Am. J. Clin. Pathol. 57: 820–828, 1972.
12. Cooney, M. K. Selection of cell culture systems for virus isolation. Am. J. Med. Technol. 98:941–947, 1967.
13. Sever, J. L. Application of a microtechnique to viral serological investigations. J. Immunol. 88:320–329, 1962.
14. Pauls, F. P., Dowdle, W. R. A serologic study of *Herpesvirus hominis* strains by microneutralization tests. J. Immunol. 98:941–947, 1967.

15. Stalder, H., Oxman, M. N., Herrmann, K. L. Herpes simplex virus microneutralization: a simplification of the test. J. Infect. Dis. 131:423–430, 1975.
16. Nahmias, A. J., Chiang, W. T., Del Buong, I., Duffey, A. Typing of *Herpesvirus hominis* strains by a direct immunofluorescent technique. Proc. Soc. Exp. Biol. Med. 132:386–390, 1969.
17. Hanna, L., Keshisyan, H., Jawetz, E., Coleman, V. R. Diagnosis of *Herpesvirus hominis* infections in a general hospital laboratory. J. Clin. Microbiol. 1:318–323, 1975.
18. Taber, L. H., Brasier, F., Couch, R. B., Greenberg, S. B., Jones, D., Knight, V. Diagnosis of herpes simplex virus infection by immunofluorescence. J. Clin. Microbiol. 3:309–312, 1976.
19. Forghani, B., Schmidt, N. J., Lennette, E. H. Solid phase radioimmunoassay for identification of *Herpesvirus hominis* types 1 and 2 from clinical materials. Appl. Microbiol. 28:661–667, 1974.
20. Forghani, B., Schmidt, N. J., Lennette, E. H. Solid phase radioimmunoassay for typing herpes simplex viral antibodies in human sera. J. Clin. Microbiol. 2:410–418, 1975.
21. Benjamin, D. R. Rapid typing of herpes simplex virus strains using the indirect immunoperoxidase method. Appl. Microbiol. 28:568–571, 1974.
22. Avrameas, S. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry 6:43–52, 1969.